Research Article

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Quality Assessment Of Biosurfactants From Palm, Groundnut And Melon Oils

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Abstract

This study was carried out to establish the ability of spilled oils on soil to produce biosurfactant from fungal isolates. The bioprocess was conducted with two fungal isolates after several culturing and subculturing which was identified as Mucor spp and Fusarium spp. The research was conducted using the following medium, groundnut oil, palm oil, melon oil supplemented with spent vegetable oil as carbon sources and yeast extract which serves as a source of nitrogen. A portion of the pure culture was inoculated into the prepared broth and incubated with constant agitation for 21days (300C). The biosurfactant broth produced was then extracted by centrifugation for 10mins at 3000rpm. The supernatants were used for the emulsification index (EI) and oil displacements assay (ODA) among other biochemical test such as iodine test, saponification test and biodegradability test using (2, 6-DCPIP). The EI assay is indicative of biosurfactant concentration which gave the following trends considering the effectiveness/suitability of the fungi for the substrate in kerosene emulsion. The EI after 24hours showing the follow trend in biosurfactant emulsification activities by Mucor spp from carbon sources groundnut oil, melon oil and palm oil in kerosene emulsion as 63.63±0.64%, 60.46±0.01, and 52.2±0.2% respectively. In like manner, the E24 from biosurfactants produce by Fusarium spp from the same sequence of substrate includes 46.7±0.13%, 45.83±0.1% and 52.2±0.2%. The EI was conducted in diesel but shows a decrease. This is indicative of the concentration of biosurfactant of the fungi capable of exhibiting great activities in such substrates. The result of the ODA showed a large area displaced by the produced biosurfactant. The biosurfactant produced by Fusarium spp from palm oil sample displaced an area of 12.57 Cm2 equally with biosurfactant released by Mucor grown on groundnut oil. The least ODA was observed in melon oil for Fusarium spp. From this study the following can be deduced; the produced biosurfactants generally can be used for the remediation of kerosene contaminated materials and environments. In terms of substrate selection, groundnut oil is a better substrate which produces more and effective biosurfactant. Furthermore, comparing between the two screened fungi, the Mucor spp produces higher concentration of biosurfactant while Fusarium spp and Mucor spp both had good activities as well as high quality dispersion and Emulsification actions.

Keywords: Biosurfactant, Dispersion, Emulsion, Fungi

Introduction

Environmentally friendly microbial products such as biosurfactants with confirmed abilities for removing petroleum hydrocarbon contaminants from drill cuttings and hydrocarbon-contaminated waste streams (such as soil and refinery wastewater) is projected to be the replacement for the chemically produced detergents. They are highly favorable due to their high biodegradability, low toxicity, low environmental impact, high specificity, high stability and activity at extreme conditions, biosurfactants have wide range of industrial applications, and structural diversity [1]. Biosurfactants are a diverse and heterogeneous group of microbial metabolites synthesized by bacteria, fungi and yeasts [2]. Biosurfactants are considered eco-friendly due to their biodegradability a properly not tenable in chemical surfactants. Available information shows that the production, environmental quality and specialized used of biosurfactant is fast graining acceptance in the oil and environmental reclaiming industries [3]. The carbon source plays an important role in the growth and production of biosurfactants by microorganisms and varies from species to species. A very low yield was found when only either glucose or vegetable oil was used for the production of a biosurfactant by T. bombicola, but the yield increased to 70 g/L when both carbon sources were provided

together [4]. Vegetable oils comprised of saturated or unsaturated fatty acids with chains of 16 to 18 carbon atoms constitute a lipid carbon source for biosurfactant synthesis [5]. Different oils are adequate substrates for biosurfactants. Babassu oil (5% v/v) with a carbon source (1% glucose w/v) is a good medium for biosurfactant production. Sarubbo et al. found that two strains of C. lipolytica (1055 and 1120) produce biosurfactants toward the final of the exponential growth phase and onset of the stationary phase [6]. According to high yields of sophorose lipids, which are biosurfactants produced by the fungi T. bombicola and C. Bombicola, have been achieved using yeast extract and urea as the nitrogen source [7]. Moreover, high yields of mannosy lerythritol lipid by Candida sp. SY16, C. lipolytica and C. glabrata have been achieved with ammonium nitrate and yeast extract. Therefore, enzymes in concert with microbial activities or fungal activities as herein studied has theoretically be known to be relevant in the production of quality biosurfactants.

Materials And Methods

Source Of Carbon/Sample Collection

Palm oil, groundnut oil, was procured from an oil selling shop opposite Federal University Wukari, Taraba State Nigeria. Melon seeds were purchased from main market Wukari, grinded and the oil extracted to a reasonable quantity. Some volumes of the oils were poured spilled on the ground for 5 days and monitored for fungal growth. Thereafter, spilled oil in soil was collected in a clean container and tightly covered and transported to the laboratory for culturing.

Microorganism

The fungi were obtained by isolation, culturing in a prepared PDA agar from the culture selection unit of the Department of Microbiology, Federal University Wukari, Nigeria. They were further characterized and sub cultured to yield pure cultures of the organisms.

Preparation of Culture Medium

Throughout the study, the assayed culture medium employed was LAB M Potato Dextrose Agar (PDA). This medium was used for the growth and maintenance of the fungal isolates. The preparation of Potato Dextrose Agar (PDA) was done according to the manufacturer recipe (39g in 1L of water). The medium was sterilized by autoclaving at 121°C and 15 psi for 20 min for complete dissolution and homogeneity. Thereafter, it was allowed to cool to temperature of between 42 and 45 °C. One capsule of chloramphenicol was added to every 500 ml of sterile cooled PDA so as to prevent bacteria growth [8]. Approximately 15 ml of the cooled amended PDA was poured into each sterile petri dish of 8.6 cm (86 mm) diameter to solidify. The petri dishes that contained the medium were incubated for 24h at ambient temperature (28 °C) to check for sterility before use as described by [9].

Isolation of Fungi

The isolation technique used by was employed in this study [10]. A small section of the various oil; palm oil, groundnut oil and melon oil showing advancing margin of change were placed on the solidified agar. The three samples were placed per plate with equal distance between them. Three replicate plates for each of the rotten samples were made. The plates were incubated at 27 ± 2°C for 7 days. Fungi associated with the oils, palm oil, groundnut oil and melon oil were observed and the frequency of isolation determined using method of [11]. Subculturing was done to obtain pure cultures of the isolates

Identification of Fungal Isolates

Subculturing of the isolates was made to obtain pure culture. The colonies growing on the plates were identified macroscopically and microscopically. Colony color, type (compact, loose, aerial hyphae), texture (velvety, cottony, coarse) shape and growth pattern were observed. Direct observation of culture under the light microscope (low power) by careful preparation of slides, staining with cotton blue-in lactophenol was done. Detailed drawings of the diagnostic features and identification manual and guides according to were used [12-14].

Production Of Biosurfactant (Bioemulsifier)

Selection Of Waste As Substrate For Fungi Production Of Bio Surfactant

Method reported by was used [15]. Biosurfactant was produced in Erlenmeyer flasks (125mL) containing 25mL of media culture (40 g/L of the waste, 4% spent vegetable oil and 10 g/L yeast extract) adjusted to pH of 5. The investigated wastes were (palm oil, groundnut oil and melon oil) and spent vegetable oil used for frying foods (SVO). The medium was sterilized by autoclaving at 121°C for 15 min and inoculated after cooling (1x104cells/mL). Flasks were incubated for 21 days (30 ± 2°C). An Erlenmeyer flask with 25mL of culture medium and 4% vegetable spent oil (v/v), with no inoculation, was used as control. Every microorganism and media (biosurfactant generation) was cultured in triplicate. Following the incubation period, the culture media were filtered in filtering membrane (cellulose filter paper) with 0.45 mm porosity coupled with a 20-mL sterilized syringe. The dependent variable in this group of experiments was the emulsification index (E24).

Assays For Biosurfactant

Emulsification index (E24)

The emulsifying index test (E24) technique described by Cooper and Goldenberg and adapted by was used. 2 mL of the filtered cell-free broth was mixed with 2 mL of spent vegetable oil (a hydrocarbon compound) in a 16 x 150 mm glass pipe thread, and the mixture was agitated for up to 2 minutes in a constant tube agitator type vortex [16] .

After 24 hours, the proportion of the emulsion formed was compared to the total volume of added hydrocarbon. The emulsification index was calculated by the following Formula:

EI (%) = (*Emulsion layer height /Total height*)×100

Where, E_{24} (%) = Emulsification Index after 24 hours etc.

Oil Drop-Collapse Qualitative Test

The test was conducted in 60 x 12 mm Petri dishes containing 3.5mL of filtered cell-free extract. An oil drop was added to the cell-free extract in triplicate and observed for 0, 1, 5, 30 min, 1 and 72hrs. The result was regarded positive when the oil drop dispersed. A total of 3.5 mL fungus-free extract and 3.5mL 1M sodium dodecyl sulfate (SDS) surfactant solution was used as negative and positive control, respectively [15].

Biodegradability test using the redox 2,6-dichlorophenol indophenol (DCPIP) indicator

The test was performed according to (1997) reported by with slight modification; in test tubes and DCPIP concentration was adjusted to 0.010g/mL [15,17]. Exactly 200µL of the DCPIP solution, 10µL of spent vegetable oil and hyphae of fungi grown in waste + oil, inoculation corresponding to 3 mm of the diameter, were added to each well and kept at 27±2°C. Medium discoloring-time measurements were taken following 24 and 48 hours. DCPIP with oil and without strain was used as a positive control and DCPIP without oil and without strain was used as negative control.

Iodine Test

Iodine test was carried out adding by 4-5drops of iodine solution into a little amount of the isolated biosurfactant and it was mixed gently [18]. The colour formation was observed.

Saponification Test

2% NaOH (2ml) solution was added to the small amount of biosurfactant and shaken well to observe formation of soap [18].

Citrate Test

A Simond citrate agar was prepared according to the manufacturer instruction and inoculates with the organism. Incubate for 24 hours at 300c and observed color change. The color change from green to blue indicate positive and no color change indicate negative result.

Catalase Test

Yeast contains an enzyme called catalase. that acts as a catalyst for the reaction that breaks down hydrogen peroxide into oxygen and water ($2H_20_2$, $2H_20$, 0_2). Oxygen is highly reactive gas.

Procedure

A drop of hydrogen peroxide was put on a slide, and a small portion of the organism was picked using the tossing pin and placed into the hydrogen peroxide. The presence of hydrogen bubbles shows catalase positive, while the absence of bubbles shows catalyst negative.

Sugar Fermentation Test

Some species of yeast breakdown starch into sugar, then indicator phenol red is added and that will indicate a change in pH due to the acid production and the presence of gas.

Procedure

The triple sugar iron agar was prepared according to the manufacturer's instruction; the inoculation was inoculated with the portion of the yeast and incubated for 24 hours to 48 hours.

Result

Observation for color change and production of gas.

Statistical Analysis

The entire assay was carried out in triplicate and the analysis was done using Statistical Package for Social Sciences (SPSS) version 20 with result presented as Mean ± Standard Deviation.

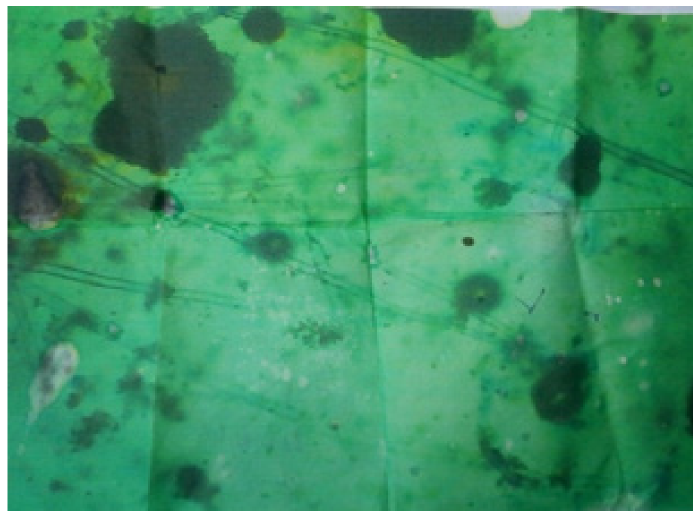

Figure 1: Organism A (*Mucor spp*)

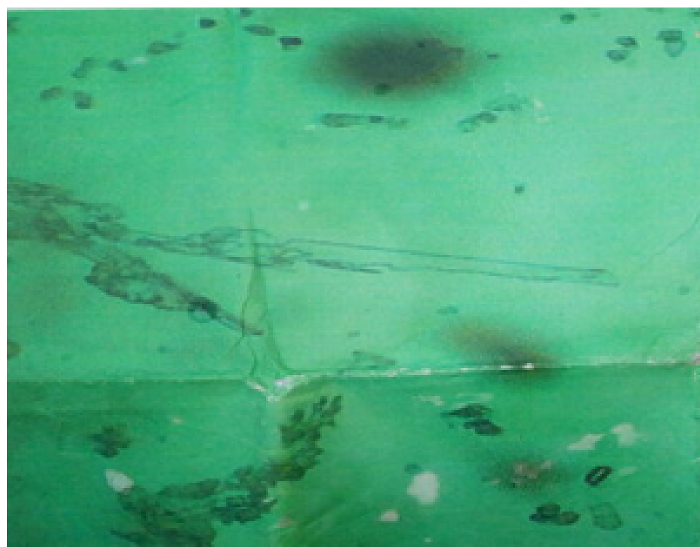

Figure 2: Organism B (Fusarium spp)

Table 1: Colony Morphology of the Biosurfactant Producing Fungi Isolates Typical Result

Feature	Fungi A (Mucor spp)	Fungi B (Fusarium spp)
Colony type	Compact	Compact
Size	1mm	1mm
Shape	Oval	Circular/Oval
Hyphae	Aerial hyphae	Aerial hyphae
Colour	Grayish brown	Light Green
Texture	Velvety	Velvety
Surface	Hairy	Hairy
Opacity	Opaque	Opaque
Diameter	73mm	75mm
Pigmentation	White	

The above colony morphology features were used for the identification of the fungi as seen in Table 1.

Table 2: Biochemical Test for Identification of Fungi

Test	Fungi A (*Mucor spp*)	Fungi B (*Fusarium spp*)
Citrate test	+	+
Catalase test	+	+
Peptone/Covax Test	_	_
Triple Sugar Identification (TSI): Glucose	+	+
Lactose	_	_
Sucrose	_	_
Hydrogen sulphide	_	_

Further identification of the fungi was the Biochemical Test shown in Table 2 above.

Table 3: Results of Iodine Test, Saponification Test and Biodegradability Test

Fungi A (Mucor spp) & B(Fusarium spp) in Different Oil Substrates	Iodine test	Saponification Test	Biodegradability Test (using 2,6-DCPIP)
Biosurfactant produced by *Mucor spp* **in Palm oil (BMP)**	_	+	+
Biosurfactant produced by *Mucor spp* in Melon oil (BMM)	_	+	+
Biosurfactant produced by *Mucor spp* in Groundnut oil (BMG)	_	+	_
Biosurfactant produced by *Fusarium spp* in Palm oil (BFP)	_	+	+
Biosurfactant produced by *Fusarium spp* in Melon oil (BFM)	_	+	+
Biosurfactant produced by *Fusarium spp* in Groundnut oil (BFG)	_	+	_

Biodegradation, Iodine and Saponification tests different biosurfactants produced is shown in Table 3 above.

Table 4: Results of Oil Drop Collapse Qualitative Test

Time	Biosurfactants From Substrates					
Time	BMP	BMM	BMG	BFP	BFM	BFG
0mins	_	_	_	_	_	_
1mins	_	_	_	_	_	_
5mins	_	_	_	_	_	_
30mins	_	_	_	_	_	_
72hrs	_	+	+	+	+	+

The oil drop test, which involved the dispersion of the oil by the produced biosurfactant showed positive result in 72 hours. Thus, it shows that time is required for dispersion/dislodge of oil or oily waste by all the biosurfactants except for the Biosurfactant produced by Mucor spp in Palm oil (BMP)

Table 5: Results of Emulsification Index (For E24, E48, E72)

BIOSURFACTANTS	KEROSINE (%E24)	DIESEL (%E24)	KEROSINE (%E48)	DIESEL (%E48)	KEROSINE (%E72)	DIESEL (%E72)
BMG	63.63±0.64[f]	54.54±0.17[d]	63.63±0.02[f]	55.02±0.03[d]	62.50±1.50[e]	62.95±0.73[e]
BFG	46.70±0.13[b]	48.56±0.06[c]	44.44±0.00[c]	42.85±2.62[b]	41.86±0.22[b]	48.57±0.01[c]
BMM	60.46±0.01[e]	39.47±1.06[a]	37.20±1.95[b]	42.92±2.01[b]	48.07±2.17[d]	45.45±0.10[a]
BFM	45.83±0.10[a]	46.67±0.25b[c]	46.00±0.99[c]	43.33±0.19[b]	42.87±0.74[b]	46.66±0.27[b]
BMP	50.00±0.50[c]	43.90±5.29[b]	51.92±0.18[d]	39.13±0.88[a]	32.55±0.14[a]	51.50±1.02[d]
BFP	52.20±0.20[d]	46.42±0.41[c]	22.00±0.20[a]	46.42±0.45[c]	44.89±0.15[c]	46.66±0.25[b]

Result represent Mean ± Standard Deviation (n=3). Mean carrying different alphabet within column are statistically significant. **BMG** represents biosurfactant produced from *mucor spp*.in groundnut oil broth. **BFG** represents biosurfactant from *Fusarium spp* grown in groundnut oil broth. **BMM** represents biosurfactant produced from *mucor spp* in melon oil broth. **BFM** represent biosurfactant, produced from *Fusarium spp* in melon oil broth, **BMP** represent biosurfactant produced from *mucor spp* in palm oil broth, **BFP** represent biosurfactant produced from *Fusarium spp* in palm oil broth.

Table 6: Table of Oil Spreading Test

Biosurfactants	BMG	BFG	BMM	BFM	BMP	BFP
Area Displaced in Oil (Cm^2)	12.57±0.55[c]	7.07±0.02[c]	9.62±0.18[d]	4.9±0.1[a]	5.72±0.19[b]	12.57±0.02[e]

Result represent Mean ± standard deviation (n=3). Mean carrying different alphabet within row are statistically significant. BMG represents biosurfactant produced from mucor spp.in groundnut oil broth. BFG represents biosurfactant from Fusarium spp grown in groundnut oil broth. BMM represents biosurfactant produced from mucor spp in melon oil broth. BFM represent biosurfactant, produced from Fusarium spp in melon oil broth, BMP represent biosurfactant produced from mucor spp in palm oil broth, BFP represent biosurfactant produced from Fusarium spp in palm oil broth.

Discussion

The tables above show the result for biosurfactant production using three different samples of palm oil, groundnut oil and melon oil that has been spilled for a very long period of time, showing progressive fungi growths which were cultured and subcultured. Two different fungal isolates from the samples were identified as *Mucor* and *Fusarium spp* from the microscopic examination of stained samples (Figs. 1 and 2). The identification followed several microbiological and biochemical tests Tables 1 and 2. These two fungi isolate shows biosurfactant producing ability to an extent. The test for oil spreading diameter and displacement area was carried out in all culture supernatant using expired engine oil. This observation showed promising biosurfactant activities (especially its surface and interfacial activity) (Table 6).

The oil spreading test is specifically an indication of the surface and wetting activities of biosurfactant (Table 6). It also indicates that Biosurfactant of *Mucor spp* on groundnut oil and *Fusarium spp* palm oil (BMG and BFP) carbon source medium has the highest surface activity (with displaced area of 12.57±0.55cm^2 and 12.57±0.02cm^2) while biosurfactant of *Fusarium spp* grown on melon oil (BFM) substrate showed the least surface activity of

about (4.90±0.10 cm^2) displaced area. This result of oil spreading or oil displacement test shows that the cell free supernatant of all the fungi isolates generates clear zones and oil displacement area as indication of biosurfactant production and activity.

Emulsification index is one of the basic characteristics of biosurfactants with which they form stable emulsion with large or small hydrocarbons e.g., kerosene and diesel as used in this research. The result of the emulsification index as presented in the table 5 reveals that kerosene and diesel form biosurfactant from both fungal isolates and was able to form stable emulsion. The highest was from biosurfactant of *Mucor spp* grown on groundnut oil (BMG) (63.63±0.64%, 63.63 ± 0.02 % and 62.50 ± 1.50 %) which was kept for 24hrs, 48hr and 72hrs using kerosene oil. Also, the emulsification activity (62.95 ± 0.73 %) of BMG in diesel after 72 hours was high. In both the two treatments (diesel and kerosene) for the formation of stable emulsion, kerosene tends to be degraded more than diesel.

Biosurfactant from *Mucor spp* in groundnut oil substrate in comparison with other sample used in this study showed the best biosurfactant activity in forming better emulsion. *Mucor spp* showed a better biosurfactant producing ability than *Fusarium spp*. The overall lowest emulsification index estimated after the three days (24hrs, 48hrs and 72hrs) was that of *Fusarium spp* grown on palm oil (BFP) sample culture showing regressive percentages of 50±0.5 %, 22±0.2%, 44.89±0.15 % after 24hrs, 48hrs and 72hrs respectively in kerosene.

Generally, the emulsification indices are due to the concentration of the biosurfactant in each of the different medium.

The emulsification index in this work showed a positive correlation with the concentration of the biosurfactant in solution. This is similar to the finding of Rahman [19].

All the biosurfactants tested negative for iodine and positive for the production of soap (saponification test) (Table 3). *Mucor spp* grown palm oil showed a severe reaction with a redox 2, 6-dichlorophenolindolphenol while a mild reaction was observed for others. The biosurfactant produced from both fungi show a mild oil drop collapse qualitative test after 72hrs. This is a clear indication that the biosurfactant is from lipid origin and has moderate surfactant activities.

Conclusion

In this research, biosurfactant producing fungi were isolated and assay using agrowaste (oils) spilled on the soil. The oil displacement and emulsification activity were performed for the actual presence of biosurfactants released by these organisms either through their secondary or primary metabolism. *Mucor spp* and *Fusarium spp* were determined. Larger concentration of biosurfactant produced was observed in the Mucor species whereas both the *Mucor* and *Fusarium spp* showed effective biosurfactant activities. Thus, these types of biosurfactants will be of great usefulness in cleaning of hydrocarbon ipso facto oil related wastes/spills.

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